Original Article

Growth-promoting effects of the hydrogen-sulfide compounds produced by *Desulfovibrio desulfuricans* subsp. *desulfuricans* co-cultured with *Escherichia coli* (DH5α) on the growth of *Entamoeba* and *Endolimax* species isolates from swine

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Summary   Certain *Desulfovibrio* sp. (anaerobic sulfate-reducing bacteria) are indigenous to swine cecum and colon, which are also common habitats for parasitic amoebae such as *Entamoeba polecki* and *Entamoeba suis*. In this study, we evaluated the growth-promoting effects of *D. desulfuricans* co-cultured with *Escherichia coli* (DH5α) and its products [*e.g.*, hydrogen sulfide (H_2S) and certain iron-sulfide (FeS) compounds] using Robinson's medium, on the 4 amoeba isolates from swine-*Entamoeba polecki* subtype (ST)-1, *E. polecki* ST-3, *Entamoeba suis*, and *Endolimax* sp., and, consequently, a continuous culture system for these amoebae was established. However, this novel culture system was required to regulate the excess H_2S dissolved in the medium by increasing air space as amoeba isolates thrive only in large air spaces (30-40%). The effects of air space and H_2S and FeS compounds on the growth of *E. polecki* ST-1 (TDP-5) were determined. *E. polecki* ST-1 (TDP-5) thrived well in culture bottles with an air space of 30-40% (aerobic) (H_2S: ~250-400 μmoles/L), but did not grow at all in an air space < 5% (microaerobic) (H_2S:~800 μmoles/L) and in anaerobic vessels (H_2S: 20-30 μmoles/L). In both H_2S-depleted and FeS compound-depleted conditions, the amoebae sp. could not thrive either. It was hypothesized that an appropriate concentration of H_2S and FeS compounds might function as important physiologically active components of electron carriers such as FeS and ferredoxin.

Keywords: Swine amoeba isolate, Robinson's medium, *Desulfovibrio desulfuricans*, hydrogen-sulfide, iron-sulfide compound

1. Introduction

Certain *Desulfovibrio* sp. (anaerobic sulfate-reducing bacteria) are bacteria indigenous to the cecum and colon of swine (*1*), which are also common habitats for parasitic amoebae, such as *Entamoeba polecki* and *Entamoeba suis* (*2-4*). Hydrogen sulfide (H_2S) produced by some enteric bacteria (*e.g.*, *Desulfovibrio* sp., *Clostridium* sp., *etc*.) is sometimes toxic to the aerobic organisms which possess the mitochondrial cytochrome c oxidase enzyme. This is because H_2S binds with iron in the cytochrome c oxidase and prevents the aerobic respiration by mitochondria. However, micro quantities of H_2S are also recognized for their cytoprotective and antioxidant activities along with the physiological

effects on energy metabolism, signal transduction, and neurotransmission. (*5-7*).

Culture strains [*Entamoeba polecki* (subtype (ST)-1 and ST-3), *Entamoeba suis*, and *Endolimax* sp.] were previously isolated from swine using Robinson's medium supplemented with a *Desulfovibrio* sp. derived from these animals. During the culture, ferrous sulfide (FeS)-like black colored precipitate was sometimes observed in the culture media in which amoebae of those isolates thrived. Hence, in the present study, we investigated the growth-promoting effect on *E. polecki* (subtype 1: TDP-5), which exhibits the highest rate of proliferation among the four isolates and is a zoonotic species infecting humans (*8*), and used the reference strain *Desulfovibrio* sp. [*Desulfovibrio desulfuricans* subsp. *desulfuricans* (NBRC13699)] instead of a *Desulfovibrio* sp. derived from swine.

2. Materials and Methods

2.1. *Amoeba isolates from swine*

The present study used amoebae isolated from swine bred at the Kitasato University School of Veterinary Medicine (Towada, Aomori, Japan), and the study was conducted according to the protocols approved by the Institutional Care and Use Committee of Kitasato University (Approval number: 17-123). The isolated culture strains were *E. polecki* ST-1 (TDP-5), *E. polecki* ST-3 (TDP-1; synonym: *Entamoeba struthionis*), *E. suis* (TDP-3), and *Endolimax* sp. (TDP-2). *E. polecki* ST-2 (SZM-1; synonym: *Entamoeba chattoni*) isolated from the stool of a wild monkey (*Macaca fuscata fuscata*) in Shizuoka, Japan, was used as a reference strain of *E. polecki* species that is not indigenous to swine.

2.2. *Robinson's medium and supplements*

Robinson's, R (defined medium for *Escherichia coli*) and BR (R medium precultured with *E. coli*) media were prepared as described by Robinson (*9*). The sulfate-reducing bacterium *Desulfovibrio desulfuricans* subsp. *desulfuricans* (NBRC 13699; NITE Biological Resource Center, Japan) and *E. coli* (type B: DH5α) (Ec) were individually cultured in a modified medium (M-ATCC 207) designed based on ATCC 210-modified Starkey's medium C (ATCC 207 (2016); American Type Culture Collection, USA) (*10*) and R medium by Robinson, respectively (*9*). The modified medium was prepared as follows. The following components were dissolved in 900 mL ultrapure water and brought to a final volume of 1 L: 5 g NaCl, 2 g citric acid monohydrate, 0.5 g KH_2PO_4, 0.1 g $(NH_4)_2SO_4$, 50 mg $MgSO_4 \cdot 7H_2O$, 1 g Na_2SO_4, 0.1 g $CaCl_2$, 50 mg ferrous ammonium sulfate, 4 mL lactic acid, 75 mg L-cysteine hydrochloride, 1 g yeast extract, and 1 mg

bromothymol blue. The pH was adjusted to 7.5 using approximately 7.5 mL of 10 N NaOH. After filter-sterilizing the medium, 20-mL aliquots were added to 50-mL culture flasks. *D. desulfuricans* (Dd) and Ec grown in this medium in anaerobic and aerobic conditions, respectively, were mixed at equal volumes in a baffled polycarbonate Erlenmeyer culture flask (Falcon; BD Biosciences, Durham, NC, USA) and cultured at 35.5°C for 2 days. The flask lid was closed and the flask was incubated at 24-27°C, and used within 2 weeks. A suspension containing Dd co-cultured with Ec (Dd/Ec) (H_2S: approximately 1,500 μmoles/L) was added just before the subculture of the amoebic isolates, as a supplement. Anaeropack-Anaero (Mitsubishi Gas Chemical Co., Inc., Chiyoda-ku, Tokyo, Japan) was used to absorb oxygen in the closed vessel. Glass vials (SV-8, 8 ml (exact capacity: 9.5 mL); Nichiden-Rika Glass Co., Ltd., Kobe, Hyogo, Japan) with screw caps, silicon packing, and sloped saline agar (4 mL) were used to cultivate the amoebic isolates.

The FeS-rich compound was obtained from the cultured medium of Dd/Ec by centrifugation (275 × g, 5 min). The precipitate was sterilized with 5 mL of 2N NaOH for over 24 hours. The precipitate was washed with 8 mL of Hanks' balanced salt solution (HBSS), pH7.4, with 0.002% phenol red, by centrifugation (275 × g, 5 min) for 3 times, and finally adjusted to approximately 0.5% (vol/vol) suspension with HBSS.

2.3. *Culture of amoebic isolates*

After establishing the amoeba strains, Dd/Ec (100 μL) was added to 1.5 mL of fresh Robinson's medium, and inoculated with amoeba suspension (1 mL), for subculture. Then, 1 mL of amoeba suspension was aspirated after mixing well and was added back to the first culture medium (final air space: ~37.2%).

The H_2S detectable SIM medium (Nissui Pharmaceutical Co., LTD, Taito-ku, Tokyo, Japan) was used to determine the viability of Dd in Dd/Ec under aerobic culture conditions.

The concentration of H_2S produced by Dd was assayed quantitatively using a HSip-1 kit (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) and a multimode microplate reader (Cytation 5, BioTek; BioTek Japan, Taito-ku, Tokyo, Japan).

Partially purified FeS-rich compounds derived from Dd/Ec were also supplemented, instead of Dd/Ec, under the existence of sufficient H_2S produced by Dd which was spontaneously proliferated and subcultured along with amoeba. The concentration of the FeS-rich compounds in the medium as a supplement was determined by referring to a study on the toxic effects in axenic culture of *Entamoeba dispar* (AS16IR) (*11*). This study was chosen as the growth of AS 16 IR is more easily affected by toxic substances than that of other axenic amoebic culture strains.

2.4. *Trichrome staining*

Trichrome staining was carried out according to the protocol described by Wheatley (*12*). However, a basic solution of Kohn's stain was used as a fixative instead of Shaudin's fixative, which contains mercuric chloride (*13*).

2.5. *DNA isolation and amplification by polymerase chain reaction (PCR)*

DNA was isolated from approximately 1×10^5 - 2×10^5 amoeba cells suspended in 100 μL of 10 mM phosphate-buffered saline (pH 7.4) using a Cica Geneus total DNA prep kit for tissue (Kanto Chemical Co., Chuo-ku, Tokyo, Japan). DNA samples from the isolates of *E. polecki*, *E. suis*, and the *Endolimax* species were used as templates for the PCR amplification of 18S ribosomal RNA (rRNA) genes. Three primer sets each for *E. polecki* ST-1 and ST-3 (Ent-1F and Esuis-1R, Epol-2F and Epol-2R, and Epol-3F and Ent-3R), *E. polecki* ST-2 (Ent-1F and Echat-2R, Echat-1F and Epol-2R, and Epol-3F and Ent-3R), and *E. suis* (Ent-1F and Esuis-1R, Esuis-2F and Esuis-2R, and Esuis-3F and Ent-3R), as well as a separate primer set (Ent-1F and Ent-3R) for the *Endolimax* species, were used (Table 1). Amplification was performed in a 20-μL reaction volume containing 2 μL of 10× Ex Taq buffer, 4 μL of 2.5 mM of each dNTP, 2 μL of each primer (10 μM; Table 1), 0.2 μL of 5 U/μL Ex Taq (Takara Bio, Inc., Kusatsu, Shiga, Japan), and 9.8 μL of H₂O. The following cycle parameters were used: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products of the suspected *Endolimax* species were used to obtain clones of the *Endolimax* 18S rRNA gene using a Qiagen PCR cloning kit (Qiagen, Com., Venlo, Netherlands).

2.6. *Sequencing and phylogenetic analysis*

The PCR products were directly sequenced using an ABI Prism BigDye Terminator v3.1 cycle sequencing ready reaction kit and an ABI Prism 3500 genetic analyzer (Applied Biosystems Japan, Ltd., Chuo-ku, Tokyo, Japan). Multiple alignment and phylogenetic analysis of the 18S rRNA gene sequences of the *Entamoeba* and *Endolimax* species were performed using ClustalW and the maximum-likelihood (ML) method using the MEGA version 6 software, respectively (*14,15*). The ML tree was derived using the Tamura-Nei model employing estimates of the proportion of invariable sites and the gamma distribution with five rate categories. Statistical significance was evaluated by bootstrapping with 1,000 replicates. The ML tree data files were visualized using MEGA version 6.

2.7. *Measurement of the individual cysts and trophozoites of each species of amoebic isolate*

The long and short diameters (l.d. and s.d.) of the individual cysts in fresh stool suspensions in deionized water and those of trophozoites isolated using the culture system were measured on a screen display using a microscope digital camera and the measuring tool of imaging software (cellSens, ver. 1.7.1; Olympus Co., Shinjuku-ku, Tokyo, Japan). The mean values were calculated from measurements of 50 trophozoites and cysts of each amoebic isolate.

Table 1. Oligonucleotide primers used for PCR

Species	Primers		Primer sequence (5′–3′)	Positions	Accession no.
Entamoeba polecki	Ent-1F	Forward	GTTGATCCTGCCAGTATTATATG	7 – 29	AF149913
ST-1 and ST-3	Esuis-1R	Reverse	AAAGATGATCATGGATTTTCACCT	864 – 887	DQ286372
	Epol-2F	Forward	CTAATATAAAAAAAGGAGAAAGG	652 – 674	AF149913
	Epol-2R	Reverse	AGATAAAGTCTCGTTCGTTATCGGA	1287 – 1311	AF149913
	Epol-3F	Forward	AGGATTGACAGATTAATAGTTTTTTCA	1199 – 1225	AF149913
	Ent-3R	Reverse	ATCCTTCCGCAGGTTCACCTA	1950 – 1970	DQ286372
Entamoeba polecki	Ent-1F	Forward	GTTGATCCTGCCAGTATTATATG	7 – 29	AF149913
ST-2 (*Entamoeba*	Echat-2R	Reverse	TAAATAACCTTTCTCCTTTTTCTATC	660 – 685	AF149912
chattoni)	Echat-1F	Forward	AGGATTTGTTTTATAACAAGTTC	471 – 493	AF149912
	Epol-2R	Reverse	AGATAAAGTCTCGTTCGTTATCGGA	1287 – 1311	AF149913
	Epol-3F	Forward	AGGATTGACAGATTAATAGTTTTTTCA	1203 – 1229	AF149912
	Ent-3R	Reverse	ATCCTTCCGCAGGTTCACCTA	1950 – 1970	DQ286372
Entamoeba suis	Ent-1F	Forward	GTTGATCCTGCCAGTATTATATG	7 – 29	AF149913
	Esuis-1R	Reverse	AAAGATGATCATGGATTTTCACCT	864 – 887	DQ286372
	Esuis-2F	Forward	ACTCTTTTAAAGCCGTAAGGT	448 – 468	DQ286372
	Esuis-2R	Reverse	ACTACATGAATATCTTTTAGGT	1379 – 1400	DQ286372
	Esuis-3F	Forward	ATTCCGGTAACGAACGAGACTTA	1336 – 1358	DQ286372
	Ent-3R	Reverse	ATCCTTCCGCAGGTTCACCTA	1950 – 1970	DQ286372
Endolimax sp.	Ent-1F	Forward	GTTGATCCTGCCAGTATTATATG	7 – 29	AF149913
	Ent-3R	Reverse	ATCCTTCCGCAGGTTCACCTA	1950 – 1970	DQ286372

2.8. Correlation between the amount of air space and growth of E. polecki ST-1 (TDP-5)

The growth-promoting effects of Dd/Ec and FeS compounds were determined by assaying the growth kinetics of the amoebic isolates, which was done by estimating the number of amoebae in cultures using a Fuchs-Rosenthal counting chamber every 24 h.

2.9. Effect of iron-sulfide compounds on the growth of E. polecki ST-1 (TDP-5)

Prior to this experiment, the amoebae were washed with HBSS by centrifugation (275 × g, 5 min) twice for removing H_2S from the culture medium. Thereafter, the growth kinetics of the washed amoebae in different culture conditions – without Dd/Ec, with Dd/Ec (100 μL), with ferric ammonium citrate (Fe) (1 mg/mL, 100

μL) – were observed.

3. Results

3.1. Molecular identification and measurement of the cultured isolates

The 18S rRNA gene sequences of *E. polecki* ST-1 (TDP-5; LC230016), *E. polecki* ST-3 (TDP-1; LC230018), and *E. suis* (TDP-4; LC230019) were consistent with all the previous reports on these subtypes and species (*16-18*). Phylogenetic analysis results pertaining to the 18S rRNA gene sequences of the three isolates of *E. polecki* (ST-1, ST-2, and ST-3) and *E. suis* are shown in Figure 1. The macaque isolate was identified as *E. polecki* ST-2 (SZM-1; LC230017). However, 0.5% of its sequence was different from the previously reported sequence of *E. polecki* ST-2
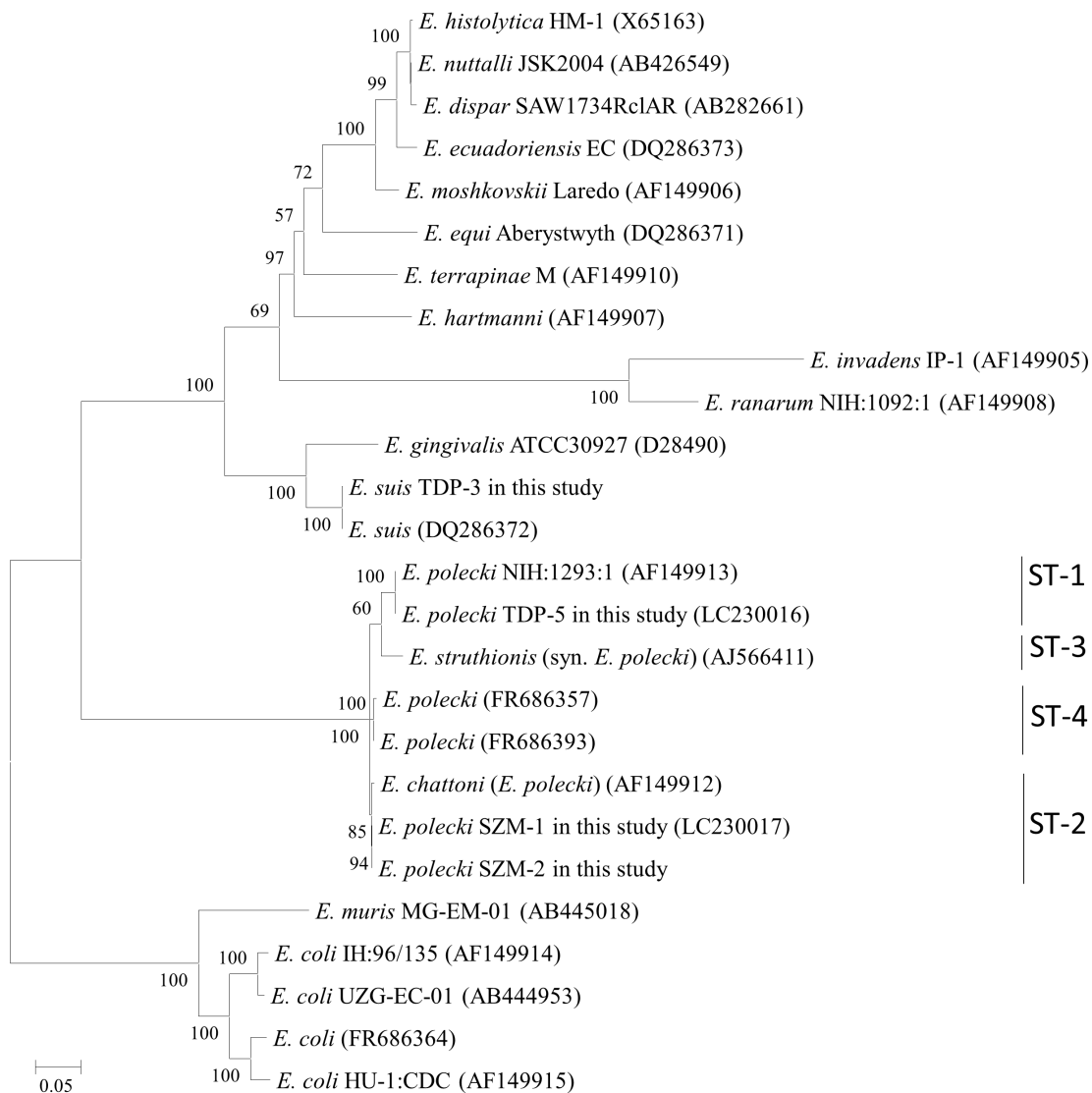

Figure 1. Phylogenetic analysis of 18S rRNA gene sequences of the three isolates of *Entamoeba polecki* (ST-1, ST-2, and ST-3) and *Entamoeba suis*. Maximum-likelihood (ML) tree derived using a Tamura-Nei model with a proportion of invariable sites and gamma distribution of 0.309 and 0.428, respectively. Significant bootstrap support (> 500) from 1,000 replicates is indicated on the left of the supported nodes. The scale bar represents the evolutionary distance for the number of changes per site. Numbers within parentheses represent the GenBank accession numbers.
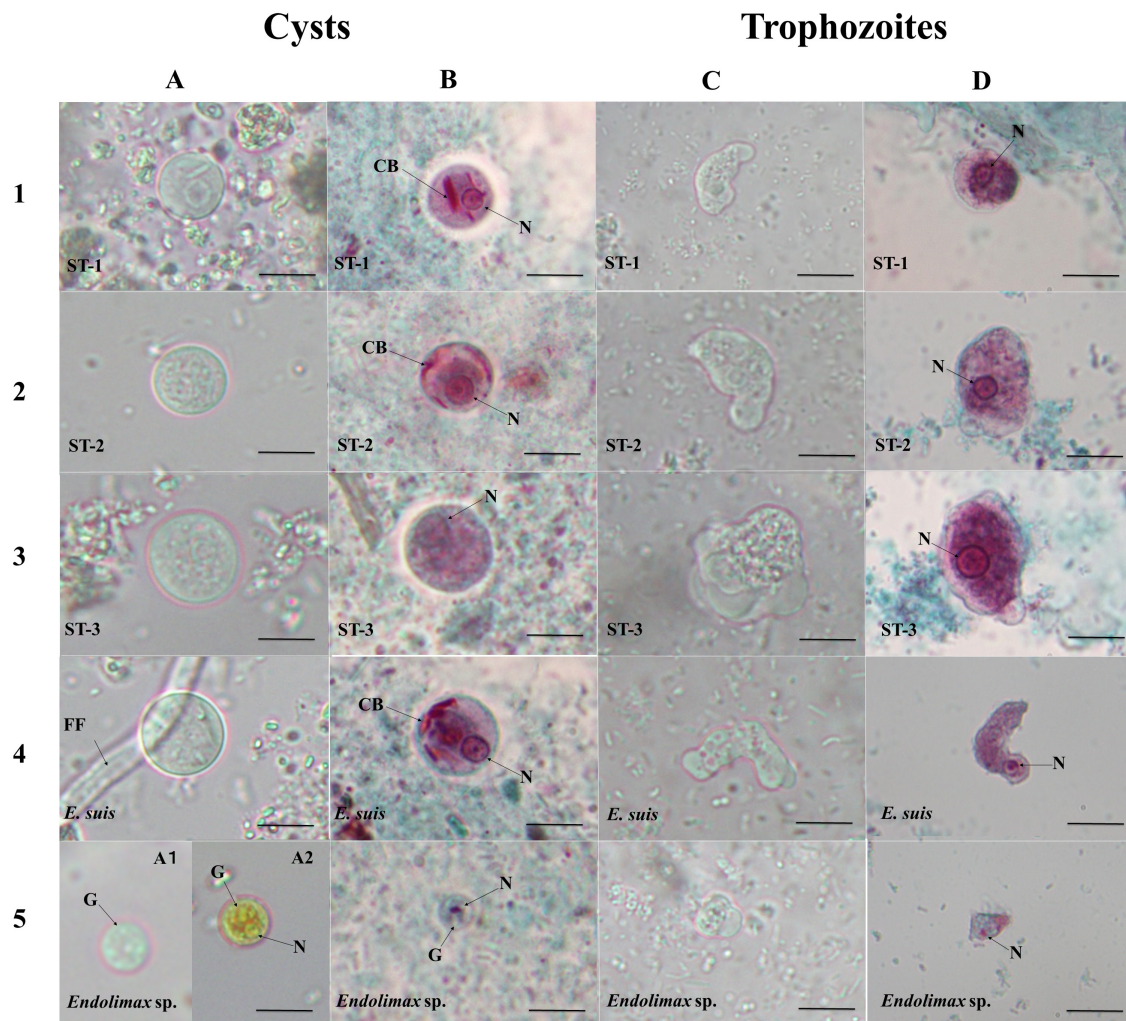
Cysts   Trophozoites

Figure 2. Light microscopy images of fresh and trichrome-stained cysts and cultured trophozoites of *E. polecki* subtypes (ST) 1, 2, and 3; *E. suis*; and the *Endolimax* species isolated from swine and a macaque. Row **1**: *E. polecki* ST-1 (TDP-5); row **2**: *E. polecki* ST-2 (SZM-1); row **3**: *E. polecki* ST-3 (TDP-1); row **4**: *E. suis* (TDP-4); row **5**: *Endolimax* species (TDP-2). Columns **A** and **A1**: fresh cysts in stool samples; column **A2**: fresh cysts stained with iodine-potassium iodide solution in stool samples; column **B**: cysts stained with trichrome dye; column **C**: live trophozoites in the culture medium; column **D**: trophozoites in the culture medium stained with trichrome dye. Scale bar = 10 μm. N, nucleus; CB, chromatoid body; FF, fungal filament; G, granule.

(AF149912) (*16*). Trophozoites of *E. polecki* ST-1 (TDP-5) with a mean length (l.d × s.d.) of 18.30 ± 3.30 × 12.85 ± 2.63 μm (Figure 2 [1C and 1D]) were isolated from small-sized uninucleate cysts with a mean length (l.d × s.d.) of 10.94 ± 1.09 × 10.38 ± 1.75 μm (Figure 2 [1A and 1B]). The latter possessed typical small rod-like chromatoid bodies of *E. polecki*. Large trophozoites of *E. polecki* ST-3 (TDP-1) with a mean length (l.d × s.d.) of 28.65 ± 6.16 × 19.56 ± 4.01 μm (Figure 2 [3C and 3D]) were isolated from uninucleate cysts with a mean length (l.d × s.d.) of 15.43 ± 2.20 × 14.14 ± 1.28 μm (Figure 2 [3A and 3B]) observed among the amoebic species. Trophozoites with a mean length of (l.d × s.d.) 23.87 ± 5.25 × 14.67 ± 3.65 μm (Figure 2 [2C and 2D]) of *E. polecki* ST-2 (SZM-1) were also isolated from the corresponding cysts with a mean length (l.d × s.d.) of 12.98 ± 1.75 × 12.39 ± 0.99 (Figure 2 [2A and 2B]). Intermediate-sized and slender trophozoites with a mean length (l.d × s.d.) of 20.58 ± 3.19 × 7.49 ± 1.04

μm (Figure 2 [4C and 4D]), which were genetically identified as *E. suis* (TDP-4) (*2*), were isolated from the corresponding cysts which were highly transparent, such that fungal filaments could be observed clearly through the cysts covering them, with mean length (l.d × s.d.) 16.13 ± 1.51 × 15.38 ± 1.34 μm (Figure 2 [4A]). The characteristics of the cysts were consistent with those of *E. suis* cysts described in the first report on this species by Clark *et al* (*2*).

Phylogenetic analysis of the five clones of the *Endolimax* species (Figure 3) showed some divergence in the 18S rRNA gene sequences (0.99-1.74%). The cause for this divergence is not clear. The 18S rRNA gene sequences of the five clones of *Endolimax* species (TDP-2; LC230011–LC230015) showed 85.3-85.8% homology with the sequence of *Endolimax nana* NIH:0591:1 (AF149916) registered in the GenBank database (*18*). The significant divergence between these clones and *E. nana* indicated that the *Endolimax* species
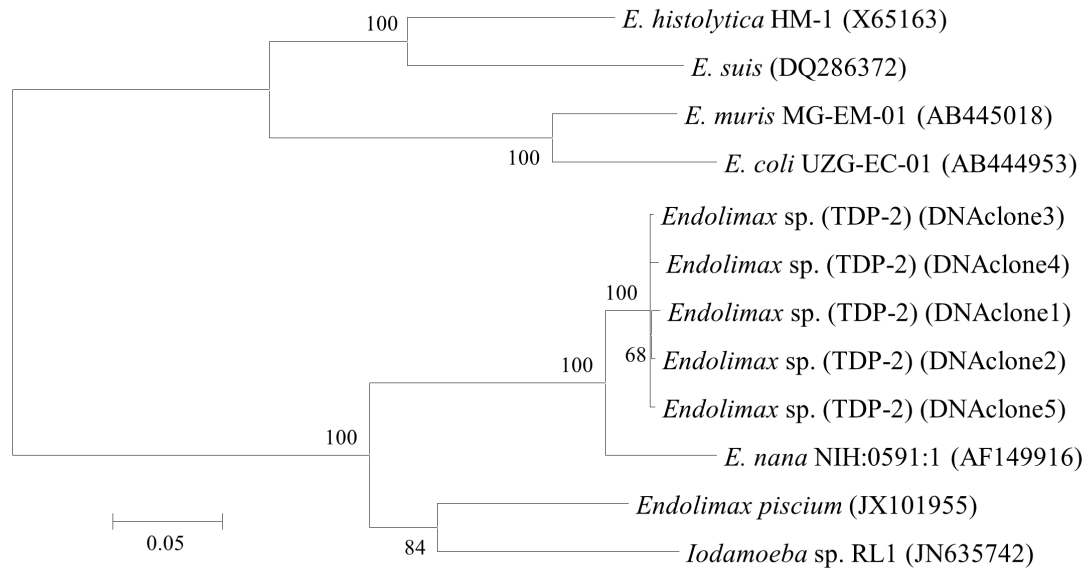
Figure 3. Phylogenetic analysis of 18S rRNA gene sequences of five clones of the new *Endolimax* species obtained by plasmid cloning. Maximum-likelihood (ML) tree derived using a Tamura-Nei model with a proportion of invariable sites and gamma distribution of 0.175 and 0.534, respectively. Significant bootstrap support (> 500) from 1,000 replicates is indicated on the left of the supported nodes. The scale bar represents the evolutionary distance based on the number of changes per site. Numbers in parentheses represent the GenBank accession numbers.

isolated in this study were not *E. nana*.

Very small trophozoites of the *Endolimax* species (TDP-2) with mean length (l.d × s.d.) 8.80 ± 0.97 × 8.15 ± 1.20 μm (Figure 2 [5C and 5D]) were also isolated from very small cysts with mean length (l.d × s.d.) 8.36 ± 1.25 × 7.41 ± 1.26 μm (Figure 2 [5A1, 5A2, and B]).

3.2. *Correlation between the amount of air space with the growth of E. polecki ST-1 (TDP-5)*

H_2S concentrations in the seven conditions examined are shown in Figure 4. The concentration of H_2S in the medium was ~800 μmoles/L, ~ 250-400 μ moles/L, and ~ 20-30 μ moles/L when the air space was < ~ 5%, ~ 30-40%, and in an anaerobic vessel, respectively. The amoebae, when supplemented with Dd/Ec (100 μL), thrived in culture bottles with an air space of 30-40% (aerobic), but did not grow at all when the air space was < 5% (microaerobic) or in anaerobic vessels (data not shown). The growth kinetics of the amoebae when supplemented with Dd/Ec (100 μL), FeS (100 μL), and Fe (50 μL) maintained in culture bottles with 30-40% air space are shown in Figure 5. The growth kinetics of the amoebae were affected by the amount of H_2S dissolved in the medium. The suitable concentration of H_2S for their growth seemed to be around ~ 250-400 μ moles/L with an appropriate amount of FeS compounds. When growth occurred in medium supplemented only with Fe, it is thought that some FeS compound was produced by reacting Fe with H_2S derived from spontaneously subcultured Dd (Dd was confirmed by H_2S-detectable SIM medium).

3.3. *Effect of iron-sulfide compounds on the growth of E. polecki ST-1 (TDP-5)*

As shown in Figure 6, the group without Dd/Ec (100 μL) did not proliferate, but the growth of Dd/Ec (100 μL) and Fe-supplemented groups drastically recovered after 3 days of culture. The results showed that amoebae could not proliferate in either H_2S- or FeS compound- depleted conditions. Surviving Dd in both culture media after 3 days from the group without Dd/Ec (100 μL) and the Fe-supplemented group was confirmed by SIM medium, whereas amoeba was recovered only in the group supplemented with Fe.

4. Discussion

We previously found that an appropriate volume (approximately 400 μL) of Dd/Ec culture suspension in BR or in complete Robinson's medium induced excystation of trophozoites from swine amoeba cysts used in this study, even after the cysts were washed with distilled water by centrifugation (275 × g for 3 min) twice (data not shown).

Based on these results, a culture system supplemented with Dd/Ec was established which enabled the first-time continuous culture of four parasitic amoebic species (*E. polecki* ST-1 and ST-3, *E. suis*, and an *Endolimax* species) from swine, and could be subcultured for over two years.

In this culture system, it was hypothesized that Dd itself, H_2S derived from Dd, and some FeS compounds produced by reaction with exogenous iron might
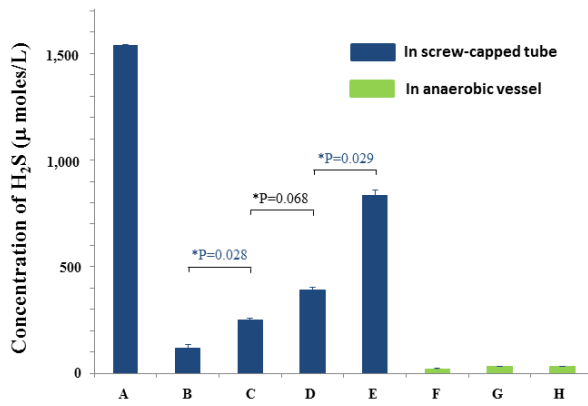
Figure 4. The concentration of H₂S in the media correlates with the seven different conditions. The concentrations of H₂S dissolved in the media were tested under various conditions as follows: **A)** Modified ATCC 207 medium cultured with Desulfovibrio desulfuricans co-cultured with Escherichia coli (Dd/Ec) [air space: 40% (AS 40%)] 7 days after cultivation at 25°C; **B)** Robinson's medium (Rm) (AS 40%); **C)** Rm + Dd/Ec (100 μL: added once without adding Dd/Ec anew during the next three subcultures) (AS 40%); **D)** Rm + Dd/Ec (100 μL: added at each time of subculture) (AS 40%); **E)** The same as **B)** except for AS, which was < 5%; **F)**, **G)** and **H)** were the same conditions as **B)**, **C)**, and **D)**, respectively, but cultured in anaerobic vessels. **B) – H)** were cultured at 35.5 °C. Data are presented as the mean and standard deviations of two measurements from each culture. Comparisons between two groups (**B and C, C and D**, and **D and E**) was made by performing a two-sided Wilcoxon's signed rank test based on the summed data of two experiments to confirm reproducibility. The differences in the medium concentrations of H₂S between **B** and **C** ($P = 0.028$) and **D** and **E** ($P = 0.029$) were confirmed as statistically significant.

Figure 6. Growth-promoting effect of *Desulfovibrio desulfuricans* co-cultured with *Escherichia coli* (Dd/Ec) and iron on *E. polecki* ST-1 (TDP-5). The number of trophozoites was counted daily for 4 days after inoculation. Data are presented as the mean and standard deviations of four measurements of each culture. **A)** Rm; **B)** Rm + Dd/Ec (100 μL); **C)** Rm + ferric ammonium citrate (1 mg/mL: 100 μL) (Fe). ***A) – C)** were cultured with AS 40% at 35.5°C. Post hoc comparisons of the number of trophozoites among those three different culture conditions were made by performing a Turkey's test (significance level, 5%). The differences in the number of trophozoites between **A** and **B** and between **A** and **C**, but not between **B** and **C**, 3 days after inoculation were confirmed to be statistically significant.
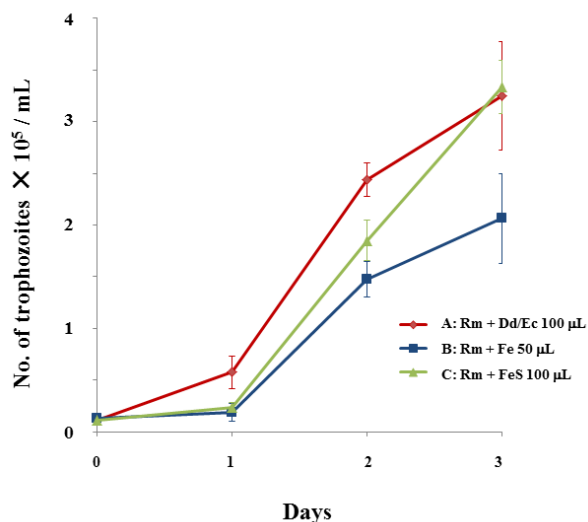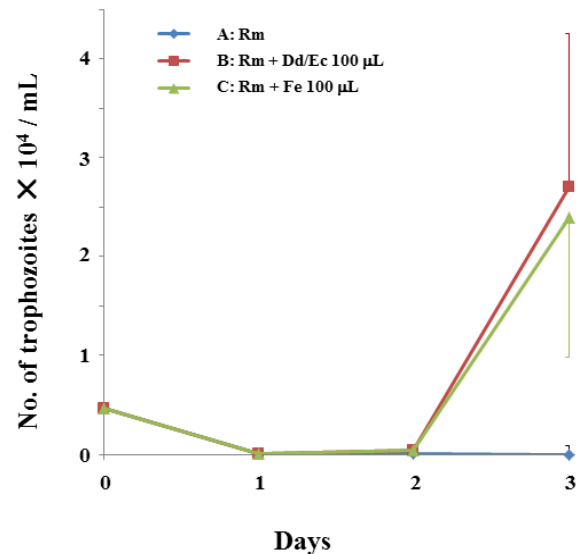

Figure 5. Growth kinetics of *Entamoeba polecki* ST-1 (TDP-5) supplemented with *Desulfovibrio desulfuricans* co-cultured with *Escherichia coli* (Dd/Ec), Fe, and partially purified FeS compounds. The number of trophozoites was counted daily for 4 days after inoculation and after subculturing seven times in the same manner at 35.5°C (AS 40%). Data are presented as the mean and standard deviations of four measurements for each culture. **A)** Rm + Dd/Ec (100 μL); **B)** Rm + ferric ammonium citrate (1mg/mL: 50 μL) (Fe); **C)** Rm + partially purified FeS (0.5% vol/vol, 100 μL). ***A) – C)** were cultured with AS 40% at 35.5°C. Post hoc comparisons of the number of trophozoites among those three different culture conditions were made by performing a Turkey's test (significance level, 5%). The differences in the number of trophozoites among **A**, **B**, and **C** on days 2 and 3 after inoculation were confirmed to be statistically significant.

function as important physiologically active compounds such as components of electron carriers (*e.g.* FeS, ferredoxin, *etc.*). These electron carriers participate in anaerobic energy metabolism (*19-21*) and are involved in the growth of the amoeba species from swine used in this study and a reference strain of *E. polecki* from macaque. This theory is further supported by an interesting report by Reeves *et al.* (*22*) which stated that ferredoxin isolated from *Entamoeba histolytica* (pathogenic *Entamoeba* species) could be converted to an apoprotein and be experimentally reconstituted with iron and H₂S.

The culture system with aerobic conditions (airspace of 30-40%) provided a suitable condition for the growth of the amoebic isolates. This might result from 1) lactic acid in R mdium; and H₂, aerobic metabolic products of Ec; and ionized H₂ produced from H₂S in the medium by reaction with sufficient oxygen from Dd itself, were available as energy sources for the growth of Dd and 2) large airspace and small liquid phase of the medium can consume the excess H₂S dissolved in the medium. Dissolved H₂S in the culture medium at this concentration (~ 250-400 μ moles/L) practically eliminated the contamination by aerobic fungi and bacteria that often inhibit the growth of amoebic isolates (data not shown).

In these culture conditions, Dd could also be

subcultured in the fresh medium along with the amoebae. However, sometimes Dd/Ec culture suspension, Fe or partially purified FeS compound from the culture suspension had to be added to the medium for successful subculture of the amoeba isolates, even though the medium already had serum iron from the Robinson's medium. *D. desulfuricans* is also known to contain a ferredoxin including the cluster [4Fe-4S] (*23*). Hence, bacterial cells might also represent a source of FeS compounds.

Indeed, a previous case report indicated that the trophozoites of *E. polecki* co-infected with *Lawsonia intracellularis* which belongs to the same family as *D. desulfuricans,* were observed in a lesion of ileitis caused by *L. intracellularis*, an irregular parasitic location (*3*).

The 18S rRNA gene sequences of the five clones of the *Endolimax* species obtained by plasmid cloning were not identical, and the reason for this is unclear. El-Sherry *et al*. (*24*) reported similar differences in the 18S rRNA gene sequences of coccidian protozoan parasites, resulting in multiple 18S rRNA gene sequences obtained from two single oocyst-derived lines of *Eimeria meleagrimitis* and *Eimeria adenoids*. Therefore, this indicates that divergent and paralogous 18S rRNA gene copies clearly exist within the nuclear genome of *E. meleagrimitis*.

The growth-promoting mechanism of this culture system could not be understood entirely. However, sulfur and iron derived from the Dd/Ec cultured medium had a critical and fast-acting effect on the proliferation of the amoeba species. Therefore, if the growth-promoting substance and its physiological activities could be elucidated, it may be utilized for further research on pathogenic mechanisms and the development of therapeutic agents against pathogenic protozoan species that possess an anaerobic energy metabolism involving iron, sulfur, and FeS compounds. While considering the difference between this limited in vitro culture condition and an actual in-vivo state in the intestine, the culture system could also be used to isolate other *Entamoeba* species such as *Entamoeba histolytica*, *Entamoeba coli*, and *Entamoeba muris* (*25*), among others.

Acknowledgements

References

1.   Butine TJ, Leedle JA. Enumeration of selected anaerobic bacterial groups in cecal and colonic contents of growing-finishing pigs. Appl Environ Microbiol. 1989; 55:1112-1116.

2.   Clark CG, Kaffashian F, Tawari B, Windsor JJ, Twigg-Flesner A, Davies-Morel MC, Blessmann J, Ebert F, Peschel B, Le Van A, Jackson CJ, Macfarlane L, Tannich E. New insights into the phylogeny of *Entamoeba* species provided by analysis of four new small-subunit rRNA genes. Int J Syst Evol Microbiol. 2006; 56:2235-2239.

3.   Matsubayashi M, Kanamori K, Sadahiro M, Tokoro M, Abe N, Haritani M, Shibahara T. First molecular identification of *Entamoeba polecki* in a piglet in Japan and implications for aggravation of ileitis by coinfection with *Lawsonia intracellularis*. Parasitol Res. 2015; 114:3069-3073.

4.   Matsubayashi M, Sasagawa Y, Aita T , Tokoro M, Haritani M, Shibahara T. First report of mixed of *Entamoeba polecki* (ST-1) and *E. suis* infection in piglets shedding abnormal feces by histopathological and molecular surveys. Acta Parasitol. 2016; 61:665-670

5.   Zheng Y, Ji X, Ji K, Wang B. Hydrogen sulfide prodrugs—a review. Acta Pharm Sin B. 2015; 5:367-377.

6.   Benchoam D, Cuevasanta E, Möller MN, Alvarez B. Hydrogen sulfide and persulfides oxidation by biologically relevant oxidizing species. Antioxidants (Basel). 2019; 8:48.

7.   Wetzel MD, Wenke JC. Mechanisms by which hydrogen sulfide attenuates muscle function following ischemia-reperfusion injury: Effects on Akt signaling, mitochondrial function, and apoptosis. J Transl Med. 2019; 17:33.

8.   Stensvold CR, Winiecka-Krusnell J, Lier T, Lebbad M. Evaluation of PCR method for detection of *Entamoeba polecki*, with an overview of its molecular epidemiology. J Clin Microbiol. 2018; 56:e00154-18.

9.   American Type Culture Collection. ATCC medium: 207 Modified Starkey's medium C. *https://www.atcc.org/~/media/8ECF033ECF304CF3B92802660F5046C8.ashx* (accessed February 02, 2018).

10.   Robinson GL. The laboratory diagnosis of human parasitic amebae. Trans R Soc Tro Med Hyg. 1968; 62:285-294.

11.   Khalifa SA, Imai E, Kobayashi S, Haghighi A, Hayakawa E, Takeuchi T. Growth-promoting effect on iron-sulfur proteins on axenic cultures of *Entamoeba dispar*. Parasite. 2006; 13:51-58.

12.   Wheatley WB. A rapid staining procedure for intestinal amoebae and flagellates. Am J Clin Pathol. 1951; 21:990-991.

13.   Kohn JA. A one stage permanent staining method for faecal protozoa. Dapim Refuiim Med Q Isr. 1960; 19:160-161.

14.   Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013; 30:2725-2729.

15.   Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994; 22:4673-4680.

16.   Stensvold CR, Lebbad M, Victory EL, Verweij JJ, Tannich E, Alfellani M, Legarraga P, Clark CG. Increased sampling reveals novel lineages of *Entamoeba*: Consequences of genetic diversity and host specificity for taxonomy and molecular detection. Protist. 2011; 162:525-541.

17.   Ponce-Gordo F, Martinez-Diaz RA, Herrera S. *Entamoeba struthionis* n. sp. (Sarcomastigophora: Endamoebidae)

from ostriches (Struthio camelus). Vet Parasitol. 2004; 119:327-335.

18. Silberman JD, Clark CG, Diamond LS, Sogin ML. Phylogeny of the genera *Entamoeba* and *Endolimax* as deduced from small-subunit ribosomal RNA sequences. Mol Biol Evol. 1999; 16:1740-1751.

19. Gillin FD, Diamond LS. *Entamoeba histolytica* and *Giardia lamblia*: Growth responses to reducing agents. Exp Parasitol. 1981; 51:382-391.

20. Diamond LS. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amebae. J Parasitol. 1968; 54:1047-1056.

21. Reeves RE, Warren LG, Susskind B, Lo HS. An energy-conserving pyruvate-to-acetate pathway in *Entamoeba histolytica*. Pyruvate synthase and a new acetate thiokinase. J Biol Chem. 1977; 252:726-731.

22. Reeves RE, Guthrie JD, Lobelle-Rich P. *Entamoeba histolytica*: Isolation of ferredoxin. Exp Parasitol. 1980; 49:83-88.

23. Zubieta JA, Mason R, Postgate JR. A four-iron ferredoxin from *Desulfovibrio desulfuricans*. Biochem J. 1973; 133:851-854.

24. El-Sherry S, Ogedengbe ME, Hafeez MA, Barta JR. Divergent nuclear 18S rRNA paralogs in a turkey coccidium, *Eimeria meleagrimitis*, complicate molecular systematics and identification. Int J Parasitol. 2013; 43:679-685.

25. Kobayashi S, Suzuki J, Takeuchi T. Establishment of a continuous culture system for *Entamoeba muris* and analysis of the small subunit rRNA gene. Parasite. 2009; 16:135-139.